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TOXICITY OF RDX, HMX, TNB, 2,4-DNT, AND 2,6-DNT TO THE EARTHWORM, *EISENIA FETIDA*, IN A SANDY LOAM SOIL

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14. ABSTRACT The U.S. Environmental Protection Agency is developing Ecological Soil Screening Levels (Eco-SSLs) for ecological risk assessment of soil contaminants at Superfund sites. Insufficient information existed to generate Eco-SSLs for explosives and related materials in soil. The earthworm (<i>Eisenia fetida</i>) reproduction test was conducted in Sassafras sandy loam soil amended with hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) to fill the data gaps. ⁵ Tests were conducted in freshly amended and in amended soils subjected to a weathering/aging process to better reflect exposure conditions in field soils. The order of toxicity in freshly amended soils, based on EC ₂₀ values for <i>E. fetida</i> juvenile production derived from non-linear regression analysis, was HMX > RDX > 2,6-DNT > TNB > 2,4-DNT. The order of toxicity of weathered/aged energetic materials in amended soils was RDX > 2,6-DNT > TNB > 2,4-DNT > HMX. Correlation of soil concentration with toxicity did not change when toxicity data were regressed with water extractable concentrations compared to acetonitrile extractable concentrations. Study results will be provided to the Eco-SSL workgroup for review and inclusion in the Eco-SSL database, and for developing Eco-SSLs for RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB.					
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PREFACE

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TOXICITY OF RDX, HMX, TNB, 2,4-DNT, AND 2,6-DNT TO THE EARTHWORM, *EINSENIA FETIDA*, IN A SANDY LOAM SOIL

1. INTRODUCTION

Many sites associated with military operations that involve munitions manufacturing, disposal, testing, and training contain elevated levels of explosives and related materials in the soil. Concentrations of explosives in soil were reported to exceed 87,000 mg kg⁻¹ for 2,4,6-trinitoluene (TNT) and 3,000 mg kg⁻¹ for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX).¹ Although these energetic materials (Ems) and their degradation products are persistent in the environment, their effects on soil biota have not been sufficiently investigated. This oversight has presented a challenge for site managers who have to distinguish between the sites that pose significant environmental risks from those that do not; prioritize contaminated sites by the level of risk posed; quantify the risks at each site; and develop appropriate remedial actions and cleanup goals.

To address this problem, the U.S. Environmental Protection Agency (USEPA), in conjunction with stakeholders, is developing benchmarks for scientifically based ecological soil screening levels (Eco-SSLs) for contaminants most frequently found at Superfund sites. The Eco-SSLs are defined as concentrations of chemicals in soil that, when not exceeded, will be protective of terrestrial ecosystems from unacceptable harmful effects. The Eco-SSLs are needed to identify contaminant explosive levels in soil that present an unacceptable ecological risk. These benchmarks can be used in a screening level Ecological Risk Assessment (ERA) to identify those contaminants in soil that warrant additional evaluation in a baseline ERA, and to eliminate those that do not. Eco-SSLs are derived using published data generated from laboratory toxicity tests with different test species relevant to soil ecosystems. After an extensive literature review, the Eco-SSL workgroup determined that there was insufficient information for explosives to generate Eco-SSL benchmarks for soil invertebrates.²

The objective of this study was to meet specific criteria and produce benchmark data for the development of an Eco-SSL for RDX, HMX, 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) for soil invertebrates:²

(a) Tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of energetics.

(b) Experimental designs for laboratory studies were documented and appropriate.

(c) Both nominal and analytically determined concentrations of chemicals of interest were reported.

(d) Tests were conducted with both negative and positive controls.

- (e) Chronic or life cycle tests were used.
- (f) Appropriate chemical dosing procedures were reported.
- (g) Concentration-response relationships were reported.
- (h) Statistical tests used to calculate the benchmark and level of significance were described.
- (i) The origins of test species were specified and appropriate.

Several soil invertebrate toxicity tests, for which standardized protocols have been developed, can effectively be used to assess the toxicity and derive the protective benchmark values for EMs.³⁻⁷ In this study, the Earthworm Reproduction Test was used. This test was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and because of its inclusion of at least one reproductive component among the measurement endpoints.

Special consideration in assessing chemical toxicity for Eco-SSL development was given to the effects of the weathering and aging of contaminant explosives in soil, which commonly occurs at contaminated sites. Weathering/aging of chemicals in soil may reduce exposure of soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption, precipitation, immobilization, occlusion, microbial transformation and other fate processes. The result may be a dramatic reduction in the amount of chemical that is bioavailable, compared to tests conducted with freshly-amended chemicals or those tested following a short equilibration period (e.g., 24 hr). Additionally, degradation products, formed during the weathering and aging process, may be more toxic to soil organisms than the parent material. A weathering and aging procedure was incorporated to more closely simulate the exposure effects on soil invertebrates in the field.

2. MATERIALS AND METHODS

2.1 Soil Collection and Characterization.

The soil used in these studies was Sassafras sandy loam (SSL), a fine-loamy, siliceous, semiactive, mesic Typic Hapludult soil, collected from M-Field (a grassy field) at Aberdeen Proving Ground, MD.⁸ Vegetation and the organic horizon were removed and the top 6 in. of the A-horizon soil were then collected. The soil was sieved through a 5-mm² mesh screen; air-dried for at least 72 hr and mixed periodically to ensure uniform drying; passed through a 2-mm sieve; and stored at room temperature before being used in testing. The soil was then analyzed for physical and chemical characteristics by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD. The results of these analyses are presented in Table 1.

Table 1. Physical and chemical characteristics of SSL soil (analyzed by the Cooperative Extension Service, University of Maryland Soil Testing Laboratory, College Park, MD).

Soil Parameter	Sassafras Sandy Loam (SSL)
Sand %	69.00
Silt %	13.00
Clay %	17.00
Texture	sandy loam
CEC cmol kg ⁻¹	5.49
Organic matter %	1.30
pH	5.20

2.2 Test Chemicals.

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Chemical Abstracts Service (CAS): 121-82-4; 99%), HMX (CAS: 2691-41-0; 99%), 2,4-DNT (CAS: 121-14-2; 98%), 2,6-DNT (CAS: 606-20-2; 98%), and TNB (CAS: 99-35-4; 99.7%) were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada). Beryllium sulfate (BeSO₄·4H₂O) (CAS: 7787; 99.99%) was used as the positive control in all the tests. Acetone (CAS: 67-64-1; HPLC Grade) was used for preparing EM solutions during soil amendments. Acetonitrile (CAS: 75-05-8; HPLC Grade) was used for extractions for chemical analyses. Methanol (CAS: 67-56-1, Chromatography grade, 99.9%) was used in determinations by HPLC. Certified standards of the EMs (AccuStandard, Inc., New Haven, CT) were used in HPLC determinations. Unless otherwise specified, ASTM type I water (American Society of Testing and Materials, <http://www.astm.org>), obtained using Milli-RO[®] 10 Plus followed by Milli-Q[®] PF Plus systems (Millipore[®], Bedford, MA), was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM type II water, analytical reagent grade nitric acid 1% (v/v), and ASTM type I water.

2.3 Soil Amendment Procedures.

A soil concentrate of EM for both the range-finding and the definitive tests was prepared in separate glass volumetric flasks and dissolved in acetone. Carrier controls were treated with acetone only. The soil was spread to a thickness of 2.5 cm to increase the surface area covered by the EM. The EM/acetone solution was pipetted evenly across the soil surface, ensuring that the volume of solution added at any time did not exceed 15% (v m⁻¹) of the dry mass soil. After the addition of the EM solution, the volumetric flask was rinsed twice with a known volume of acetone and pipetted onto the soil. If the total volume of solution needed to amend the soil exceeded 15% (v m⁻¹), the solution was added in successive stages, allowing the acetone to evaporate for a minimum of 2 hr under a chemical hood. The amended soil was then air-dried overnight (minimum of 18 hr) in a dark chemical hood to prevent photolysis of the EM. Each soil treatment sample was transferred to a fluorocarbon-coated high-density polyethylene

container and mixed for 18 hr on a 3-dimensional rotary mixer. The final nominal target treatment concentrations for the definitive tests were prepared by mixing the initial soil concentrate of an EM with clean SSL soil for 18 hr on a 3-dimensional rotary mixer. After mixing, the soil was hydrated with ASTM type I water to 17.1% of the dry soil weight (95% water holding capacity (WHC); 18% water) for toxicity testing in freshly amended soils, or 60% of the WHC (10.8% dry soil wt.) for the weathering/aging procedure. The hydrated soil, prepared for the toxicity tests, was allowed to equilibrate for 24 hr before adding earthworms.

2.4 Measurement of Soil pH.

The pH of the test soils was determined at the beginning and end of each definitive toxicity test using a method adapted from the Soil Survey Laboratory Methods Manual.⁹ Five grams of ASTM type I water was added to 5 g of soil. The soil slurry was vortexed for 10 s every 5 min for 30 min. The soil slurry was then vortexed for 10 s, 1 min before pH measurement. The pH electrode was rinsed thoroughly with ASTM type I water, blotted dry, standardized with pH 4 and pH 7 buffers, rinsed, and blotted. The pH was measured in the solution above the soil surface while stirring gently until the reading stabilized. The electrode was rinsed with ASTM type I water and blotted before each measurement.

2.5 Acetonitrile Extraction of Energetics in Soil.

The EMs were extracted in triplicate from all control and treated soils at the beginning of each definitive test, using freshly amended and weathered/aged soils according to US EPA Method 8330.¹⁰ The samples for chemical analysis were hydrated for 24 hr. Ten mL acetonitrile was then added to approximately 2.0 g of soil from each treatment concentration in polypropylene 50-mL centrifuge tubes and sampled in triplicate. Soil dry fraction (dry wt./wet wt.) was determined in triplicate from subsamples of each treatment. The samples were vortexed for 1 min, then sonicated in the dark for 18 hr at 20 °C. Five mL of supernatant was transferred to a glass tube to which 5 mL of 5-g $\text{CaCl}_2 \text{ L}^{-1}$ solution was added. The supernatant was filtered through 0.45- μm PTFE syringe cartridges. Soil extracts were analyzed and quantified by HPLC. In the present report, acetonitrile soil extraction is referred to as total extraction (concentration in dry soil). Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 to 10.

2.6 ATCLP Extraction of Energetics in Soil.

Soil samples were extracted using an Adapted Toxicity Characteristic Leaching Procedure (ATCLP)¹¹ at the beginning of each definitive test to determine the water-soluble fraction of EMs in amended soils. ATCLP is a modification of the Toxicity Characteristic Leaching Procedure (TCLP) (40 Code of Federal Regulations (CFR) Part 268.41, Hazardous Waste Management, method 1311). The modification involved substitution of acetic acid for CO_2 -saturated water, which resulted in better simulating soil-water conditions due to respiration by soil biota. All extractions were done in triplicate. For each treatment concentration, 4 g of soil (dry weight) were transferred in triplicate into 20-mL vials. Sixteen mL of CO_2 -saturated water (pH 3.8 to 4.0) was added and the vials were immediately sealed. Soil samples were vortexed for 45 s and mixed in the dark for 18 hr on a rotary (end-over-end) mixer (30 rpm) at

room temperature. Settled supernatants were filtered through 0.45- μ m PTFE syringe cartridges. An equivalent volume of acetonitrile was added to filtered soil extract prior to HPLC analysis. In the present report, ATCLP soil extraction is referred to as the water-soluble fraction of EM. Nominal and determined (measured) concentrations used in the definitive tests are shown in Tables 2 to 10.

2.7 Chemical Analysis.

Soil extracts were analyzed by reversed-phase HPLC using a variation of EPA Method 8330, which was modified in 2 ways. First, the final solvent for the energetic compounds was a mixture of 60-parts water and 40-parts acetonitrile rather than a 50:50 ratio to increase peak resolution. Secondly, the flow rate of the 50:50 methanol/water mobile phase was 1.0 ml/min rather than 1.5 ml/min. A 25 cm x 4.6 mm x 5-micron particle size C-18 column was used for all determinations since only 1 energetic compound was analyzed at a time. The instrument used was a Beckman *System Gold*, consisting of a model 126-programmable solvent module, model 168-diode array detector and a model 507-automatic sampler. Calibration curves were generated before each HPLC run by dissolving certified standards (AccuStandard, Inc., New Haven, CT) of RDX and HMX in 60:40 water/acetonitrile in a range of concentrations appropriate for each run. The method detection limit was 0.05 mg kg⁻¹. Blanks and standards were placed intermittently between unknown samples.

2.8 Weathering/Aging of Energetics in Amended Soil.

All soil treatment concentrations and negative controls that were not used for the freshly amended toxicity tests were subjected to a simulated weathering/aging procedure, which consisted of alternating wetting/air-drying cycles for 90 days prior to the commencement of the definitive tests. Weathering/aging of test soils was conducted in Teflon[®]-lined steel trays in a greenhouse. Soil treatments were initially hydrated to 60%, 10% of the soil dry wt. of the WHC, then placed in the greenhouse to dry. All soil treatments were weighed and adjusted to 60% of WHC twice each week, and afterward brought to 95% of WHC for initiation of bioassays. Therefore, the soil moisture used for all toxicity tests was 17.1% of the soil dry weight.

2.9 Toxicity Assessment.

The chronic test used in this study was a 56-day reproduction test.¹² The endpoints of this test are the number of juveniles produced, the number of cocoons produced, and the adult survival rate. Guidelines for these assays were originally developed for use with artificial soil (USEPA Standard Artificial Soil); however, research for this study has shown that these tests could also be successfully conducted using natural soils.¹³

2.9.1 Principle of the Test.

Adult *Eisenia fetida* are exposed to a range of concentrations of the test chemical added to soil. The test consists of 2 steps. First, a 21-day range-finding test is conducted in which adult survival and cocoon production is assessed using up to 5-treatment concentrations and 2 replicates. The next step is a definitive 56-day test in which survival, live weight, dry weight, cocoon production, and juvenile production are assessed using a greater number of concentrations and replicates. Adult survival and cocoon production in the range-finding test are used to determine the concentration range of test chemicals used in the definitive tests. In the definitive tests, adult survivors are counted and removed from the soil after 28 days. At this time, cocoons and juveniles are harvested and also counted. Ecotoxicological parameters are derived from regression and variance analyses. These parameters include the No Observed Effect Concentration (NOEC), the Lowest Observed Effect Concentration (LOEC) and the effective concentration that causes an X% reduction in adults, i.e., ECp (e.g., EC₂₀, EC₅₀).

2.9.2 Validity of the Test.

Validity criteria include the following performance parameters for the negative controls:

- a) The mean mortality does not exceed 10% in range-finding and definitive tests.
- b) The number of juveniles per five worms is ≥ 15 .
- c) The coefficient of variation for the control reproduction is $\leq 30\%$ at the end of

the test.

2.9.3 Earthworm Culture.

Earthworms (*E. fetida*) were bred in plastic containers filled with approximately 14 kg of a 1:1 mixture of sphagnum PRO-GRO peat moss (Gulf Island Peat Moss Co., PEI, Canada) and BACCTO[®] potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.2 ± 0.1 by adding calcium carbonate (pulverized lime). The culture was kept moist at 21 ± 2 °C with continuous light. Earthworm colonies were fed biweekly with dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; OB of PA, York, PA) that were saturated with water, fermented for 2 weeks; and dried and ground to a coarse powder. The cultures were synchronized so that all the worms used in a test were approximately the same age. Adult worms (0.3 g to 0.6 g) with fully developed clitella were used for testing.

2.9.4 Test Conditions.

Earthworms were acclimated for 48 hr in the test soil. The worms were selected for uniformity and placed on moist filter paper overnight to purge their gut contents. Five worms were rinsed twice with ASTM type I water; blotted on paper towels; weighed on an analytical balance; and placed on the soil surface in each of the four 400-mL (9-cm diameter) glass canning jars. The worms were selected randomly for placement across treatments. A 2-g bolus of alfalfa food was added to each jar and covered with the soil in the jar. Plastic film was stretched over the top of the containers and secured with the screw-on rings. The metal lids were excluded to

allow light exposure. Three small holes were made in the wrap with a pushpin to allow for air exchange. The worms were incubated under a 16-hr light/8-hr dark photoperiod with a mean light intensity of $12.8 \mu\text{M m}^{-2} \text{ sec}^{-1}$ (SE = 0.67) and a mean temperature of 21.6°C (SE = 0.078).

2.9.5 Endpoint Determination.

After 28 days, the worms were removed from the containers with blunt forceps. The number of surviving earthworms in each beaker were counted and recorded. Plastic wrap and screw rings were placed on the containers as described above. After an additional 28 days, cocoons and juveniles were harvested and counted. Juveniles were induced to crawl to the soil surface by immersing the sealed containers to a level just below the soil line in a heated water bath at 41 to 43°C for 20 to 25 min. The juveniles were removed from the soil surface with blunt forceps and counted. Soil was then spread and examined under a $2.25\times$ lighted magnifier to recover any additional juveniles. The number of juveniles in each container was counted and recorded. Cocoons were recovered by gently agitating the soil in a 1-mm sieve with water until only the cocoons remained on the surface of the sieve. The cocoons were placed in water in a clear glass dish. The cocoons that floated were counted as hatched; those that sank were counted as unhatched. The cocoons were then examined under the magnifier to confirm whether they were hatched or not. The number of cocoons per container was counted and recorded.

2.10 Data Analysis.

Cocoon and juvenile production data were analyzed using nonlinear regression models.^{14,15} Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select potential models. The exponential model had the best fit for all HMX and RDX data except weathered/aged ATCLP, which fit the logistic Gompertz model. All data for 2,4-DNT and 2,6-DNT fit the logistic model. Data for the TNB tests fit the exponential model except for the test performed in freshly amended soil and extracted with acetonitrile. This test fit the logistic model. The fit of the lines generated by these models were closest to the data points, the variances were the smallest, and the residuals had the best appearance (i.e., most random scattering). The two models were

Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)] \times [C/EC_p]b)}$

Exponential model: $Y = a \times e^{([\log(1-p)] / EC_p) \times C} + b$

In both models, Y is the number of juveniles produced; a is the control response; e is the base of the natural logarithm; p is the percent inhibition/100 (e.g., 0.5 for EC_{50}); C is the exposure concentration in test soil; EC_p is the estimate of effect concentration for a specified percent effect; h is the hormetic effect parameter; and b is the scale parameter.

The EC_p parameters used in this study included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL benchmarks. The EC_{50} parameter and survival data, more commonly used in previous studies, were included to enable comparisons of the results produced in this study with the results

reported by other researchers. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) was used to determine the bounded NOEC and LOEC values for adult survival, cocoon production, or juvenile production data. Mean separations were determined using Fisher's Least Significant Difference (LSD) pairwise comparison tests. A significant level of $p < 0.05$ was used to determine NOEC and LOEC values. All analyses were performed using measured EM concentrations. Statistical analyses were performed using SYSTAT 7.0.1.¹⁶

3. RESULTS

3.1 Soil Analysis.

Measured total (acetonitrile extractable) RDX concentrations in freshly amended soils ranged from 125 to 216% of nominal concentrations $\leq 6 \text{ mg kg}^{-1}$, and 91.9 to 103% of nominal concentrations $\geq 9 \text{ mg kg}^{-1}$ (Table 2). This difference may have been due to decreased instrument accuracy at concentrations close to the method detection limit (MDL) of 0.05 mg kg^{-1} . Measured RDX water extractable (ATCLP) concentrations in freshly amended soils ranged from 44.6 to 91.4% of total concentrations due to low solubility of RDX in water (Table 2).

Table 2. Nominal and average ($n = 3$) RDX concentrations (mg kg^{-1}) (in freshly amended SSL soil used in the toxicity tests with *E. fetida*, measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.34	6.19
1.5	3.2	0.34	216.0	2.07	0.88	64.0	5.41	6.35
3.0	5.3	0.62	175.0	2.34	0.20	44.6	5.37	.
6.0	7.5	0.10	125.1	5.15	0.57	68.7	5.46	.
9.0	8.6	0.45	95.9	7.24	0.71	84.0	5.46	.
18.0	18.2	0.61	100.9	15.59	0.55	85.8	5.35	.
36.0	33.1	1.67	91.9	30.21	1.08	91.4	5.41	7.04
72.0	74.1	8.29	103.0	56.71	1.55	76.5	5.42	.
144.0	148.3	4.91	103.0	93.48	0.90	63.0	5.35	6.77

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L^{-1} .

Table 3. Nominal and average (n = 3) measured weathered/aged RDX concentrations (mg kg⁻¹) (in amended SSL soil used in toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL			BDL			5.32	5.37
6.0	6.4	1.5	105.8	5.78	0.3	91.0	5.31	5.86
9.0	8.4	1.3	93.6	7.72	0.2	91.7	5.30	6.21
18.0	15.7	0.2	87.0	13.55	0.4	86.5	5.28	6.42
36.0	30.0	0.7	83.4	29.99	0.3	99.9	5.27	6.68
72.0	56.6	3.3	78.6	54.10	2.0	95.6	5.20	6.58
144.0	61.5	2.2	42.7	55.13	2.9	89.6	5.12	6.45
300.0	254.3	8.7	84.8	100.06	2.5	39.3	5.00	6.46
600.0	527.0	4.0	87.8	93.23	1.2	17.7	5.00	6.62

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L⁻¹.

Measured RDX total concentrations in weathered/aged amended soils ranged from 42.7 to 105.8% of nominal concentrations (Table 3). Measured RDX ATCLP extractable concentrations in weathered/aged amended soils ranged from 17.7 to 99.2% of total measured concentrations (Table 3). Weathering/aging of RDX in amended soils reduced total concentrations by an average of 20% compared with total-concentrations in freshly amended soils (Table 3), whereas ATCLP extractable RDX concentrations were reduced, by an average of 7% compared with freshly amended soils.

Measured mean HMX total concentrations in freshly amended soils ranged from 86.7 to 124.9% of nominal concentrations (Table 4). Measured HMX ATCLP concentrations in freshly amended soils ranged from 8.8 to 71.9% of total concentrations (Table 4). Lower recovery at higher nominal concentrations is probably due to low solubility (approximately 5 mg L⁻¹) of HMX in water. Weathered/aged HMX total concentrations in amended soils averaged from 26.7 to 93.6% of nominal concentrations (Table 5). Weathered/aged HMX ATCLP extractable concentrations in amended soils ranged from 3.2 to 180.8% of total measured concentrations (Table 5). Percent recovery was in descending order from low to high nominal concentrations, probably due to the low solubility of HMX. Weathering/aging reduced total HMX concentrations on average by 44% compared with total concentrations in freshly amended soils (Table 5), whereas ATCLP extractable HMX concentrations were increased, on average, by 11% compared with freshly amended soils. Increase in soluble HMX was greatest in nominal concentrations between 72 and 600 mg kg⁻¹. This increase in soluble HMX may have been the result of biological and/or chemical processes occurring in the soil during the weathering/aging process.

Table 4. Nominal and average (n = 3) HMX concentrations (mg kg⁻¹) (in freshly amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.47	5.56
1.5	1.3	0.32	88.0	0.46	0.07	34.7	5.68	.
3.0	2.9	0.63	96.4	1.86	0.39	64.2	5.67	5.63
6.0	6.5	0.76	108.3	2.75	0.44	42.4	5.57	.
9.0	11.2	4.98	124.9	5.92	0.51	52.7	5.58	.
18.0	15.6	0.87	86.7	11.22	0.39	71.9	5.54	.
36.0	36.0	2.77	100.1	15.17	0.55	42.1	5.54	5.16
72.0	73.6	8.25	102.2	13.10	0.06	17.8	5.53	.
144.0	141.3	7.54	98.1	12.47	0.30	8.8	5.54	5.27

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L⁻¹.

Table 5. Nominal and average (n = 3) measured weathered/aged HMX concentrations (mg kg⁻¹) (in amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	4.97	5.91
6.0	1.6	1.41	26.7	2.89	0.580	180.8	5.13	6.55
9.0	2.8	0.46	31.4	4.38	0.550	154.8	5.02	6.23
18.0	10.8	0.58	59.8	9.07	0.740	84.2	5.29	5.97
36.0	28.9	1.31	80.2	13.11	0.15	45.4	5.35	6.80
72.0	53.5	1.79	74.3	14.64	0.66	27.4	5.25	6.41
144.0	129.3	10.90	89.8	16.43	0.62	12.7	5.32	6.43
300.0	280.3	8.67	93.4	18.96	0.34	6.8	5.29	6.85
600.0	561.7	15.24	93.6	18.03	0.46	3.2	5.39	6.61

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L⁻¹.

Measured 2,4-DNT total concentrations in freshly amended soils ranged from 48 to 86% of nominal concentrations (Table 6). Measured 2,4-DNT ATCLP extractable concentrations ranged from 19 to 84% of total concentrations (Table 6). Measured

weathered/aged 2,4-DNT total concentrations in amended soils ranged from 37 to 56% of nominal concentrations (Table 7). Measured 2,4-DNT ATCLP extractable concentrations ranged from 46 to 70% of total concentrations (Table 7). Weathering/aging reduced total 2,4-DNT concentrations, on average, by 45% and ATCLP extractable 2,4-DNT concentrations by 24% compared with respective concentrations in freshly amended soils.

Table 6. Nominal and average ($n = 3$) 2,4-DNT concentrations (mg kg^{-1}) (in freshly amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile/Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.51	5.44
2	0.95	0.2	48	0.80	0.001	84.3	5.31	5.33
4	3.0	0.3	74	1.34	0.01	45.2	5.36	5.25
8	6.5	0.4	81	2.40	0.05	37.4	5.31	5.24
12	9.9	0.5	82	4.96	0.01	50.2	5.28	5.47
24	20.3	0.3	85	3.77	0.04	19.1	5.23	6.27
48	40.9	2.6	85	8.13	0.08	20.0	5.23	6.93
64	55.0	0.5	86	33.45	0.22	61.1	5.24	6.55
80	64.7	1.5	81	43.37	0.09	67.4	5.25	7.54

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L^{-1} .

Measured 2,6-DNT total concentrations in freshly amended soils ranged from 80 to 267% of nominal concentrations (Table 8). Measured 2,6-DNT ATCLP extractable concentrations ranged from 27 to 65% of total concentrations (Table 8). Weathered/aged 2,6-DNT total concentrations in amended soils ranged from 15 to 34% of nominal concentrations (Table 9). Weathered/aged 2,6-DNT ATCLP extractable concentrations ranged from 19 to 62% of total concentrations (Table 9). Weathering/aging of amended soils reduced total 2,6-DNT concentrations, on average, by 79% compared with total concentrations in freshly amended soils, whereas ATCLP extractable 2,6-DNT concentrations were reduced, on average, by 57% compared with freshly amended soils.

Table 7. Nominal and average (n = 3) measured weathered/aged 2,4-DNT concentrations (mg kg⁻¹) (in amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.41	5.95
8	3.0	0.5	37	1.67	0.03	56	5.39	5.35
12	5.2	0.2	43	2.42	0.06	47	5.34	5.94
24	11.5	0.2	48	5.22	0.02	46	5.40	6.05
48	21.5	0.3	45	11.77	0.12	55	5.35	6.02
64	31.0	0.8	48	15.40	0.15	50	5.35	6.82
80	37.3	0.8	47	20.47	0.37	55	5.31	6.90
160	71.7	2.3	45	46.07	0.37	64	5.37	7.72
320	178.7	8.4	56	125.00	2.00	70	5.38	7.37

BDL - Below detection limit.

MDL - method detection limit = 0.05 mg L⁻¹.

Table 8. Nominal and average measured (n = 3) 2,6-DNT concentrations (mg kg⁻¹) (in freshly amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.47	5.42
2	5.3	0.1	267	1.43	0.01	27	5.43	5.31
4	7.7	0.9	191	2.18	0.01	28	5.43	5.23
8	9.4	0.3	117	3.78	0.01	40	5.32	5.22
12	12.9	0.2	108	5.83	0.04	45	5.35	5.45
24	20.0	0.8	83	10.63	0.08	53	5.49	6.34
48	40.2	2.0	84	24.84	0.04	62	5.27	6.83
64	51.1	1.0	80	32.94	0.12	65	5.25	6.67
80	64.0	1.6	80	40.50	0.11	63	5.30	7.16

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L⁻¹

Table 9. Nominal and average measured ($n = 3$) weathered/aged 2,6-DNT concentrations (mg kg^{-1}) (in amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg^{-1})	Acetonitrile extraction (mg kg^{-1})	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg^{-1})	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.27	5.05
8.0	1.2	0.02	15.0	0.23	0.01	19.0	5.39	4.96
12.0	1.6	0.02	13.0	0.42	0.03	27.0	5.29	5.36
24.0	3.7	0.08	15.0	1.46	0.06	40.0	5.39	5.54
48.0	9.5	0.12	20.0	4.30	0.09	45.0	5.42	5.15
64.0	13.9	0.12	22.0	6.63	0.08	48.0	5.37	5.02
80.0	18.1	0.20	23.0	9.64	0.36	53.0	5.31	5.32
160.0	37.4	0.98	23.0	17.43	3.27	47.0	5.33	6.27
320.0	108.3	1.45	34.0	66.87	2.22	62.0	5.38	.

BDL - Below detection limit.

MDL - Method detection limit = 0.05 mg L^{-1} .

Measured TNB total concentrations in freshly amended soils ranged from 25 to 100% of nominal concentrations (Table 10). TNB recovery was greatly reduced in treatments below nominal 64 mg kg^{-1} . Measured TNB ATCLP extractable concentrations ranged from 56 to 86% of total concentrations (Table 10). These values do not include data for 8 mg kg^{-1} nominal treatment concentration, which had TNB recovery in one (0.13 mg kg^{-1}) out of 3 replicates producing an average ATCLP extractable value of 0.043 mg kg^{-1} (Table 10). Measured weathered/aged TNB total concentrations in amended soils ranged from 3 to 88% of nominal concentrations (Table 11). Measured weathered/aged TNB ATCLP extractable concentrations ranged from 31 to 72% of total concentrations (Table 11). Weathering/aging of amended soils reduced total TNB concentrations, on average, by 43% compared with total concentrations in freshly amended soils, whereas ATCLP extractable TNB concentrations were reduced, on average, by 59% compared with freshly amended soils.

Table 10. Nominal and average measured (n = 3) TNB concentrations (mg kg⁻¹) (in freshly amended SSL soil used in the toxicity tests with *E. fetida*; measured concentrations include acetonitrile extractable (U.S. EPA Method 8330) and water extractable (ATCLP) concentration values).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0	BDL	BDL	BDL	BDL	BDL	BDL	5.34	6.51
4	2.3	0.08	58	BDL	BDL	BDL	5.46	5.47
8	2.6	0.11	32	*0.043	*0.043	*1.7	5.54	6.30
16	3.9	0.48	25	2.45	0.29	62	5.42	5.94
32	13.6	1.11	43	7.68	0.25	56	5.41	.
64	45.0	1.80	70	30.22	0.52	67	5.43	7.69
128	107.0	2.52	84	83.67	1.28	78	5.39	7.84
256	221.0	12.66	86	190.95	1.40	86	5.36	7.91
384	384.7	21.15	100	328.28	14.80	85	5.36	7.70

BDL - Below detection limit. MDL - Method detection limit = 0.05 mg L⁻¹.

* TNB was recovered in one (0.13 mg kg⁻¹) out of three replicates producing an average ATCLP extractable value of 0.043 mg kg⁻¹.

Table 11. Nominal and average measured (n = 3) weathered/aged TNB concentrations (mg kg⁻¹) (in amended SSL soil used in the toxicity tests with *E. fetida*).

Nominal concentration (mg kg ⁻¹)	Acetonitrile extraction (mg kg ⁻¹)	Standard error	Acetonitrile / Nominal (%)	ATCLP extraction (mg kg ⁻¹)	Standard error	ATCLP/ Acetonitrile (%)	Mean pH	
							start	end
0.0	BDL	BDL	BDL	BDL	BDL	BDL	5.01	5.86
16.0	BDL	BDL	BDL	BDL	BDL	BDL	5.00	5.41
32.0	1.0	0.09	3.0	BDL	BDL	BDL	5.03	5.55
64.0	19.9	0.31	31.0	6.16	0.12	31.0	4.83	5.55
128.0	78.7	1.44	62.0	41.83	0.85	53.1	4.67	6.56
256.0	191.0	6.93	75.0	112.00	1.53	58.6	4.72	7.25
384.0	302.0	3.06	79.0	174.30	2.60	57.7	4.64	7.16
512.0	411.0	4.36	80.0	271.33	3.84	66.0	4.69	6.72
768.0	674.0	18.36	88.0	487.00	4.16	72.3	4.76	6.54

BDL - Below detection limit.

MDL - Method Detection Limit = 0.05 mg L⁻¹.

3.2

Range-finding Toxicity Tests.

Both RDX and HMX had no significant effect ($p > 0.0001$) on adult survival in the range-finding tests in all treatment concentrations using freshly amended soils. Cocoon numbers were reduced by 69% ($p < 0.05$) in 10 mg kg⁻¹ RDX treatment. Twenty percent of the cocoons remained at 5,000 mg kg⁻¹ RDX compared to the control. Cocoon numbers were reduced by 60% ($p < 0.05$) in 10 mg kg⁻¹ HMX. Nine percent of the cocoons remained at 5,000 mg kg⁻¹ HMX compared to the control. Results of the range-finding test showed that 2,4-DNT significantly ($p < 0.0001$) reduced cocoon production at 10 mg kg⁻¹. No cocoons were produced at 100 mg kg⁻¹, and no adults survived at the higher concentrations. Range-finding tests with 2,6-DNT showed that cocoon production was significantly ($p < 0.01$) reduced at 10 mg kg⁻¹. There were no cocoons or adults found above 10 mg kg⁻¹. Cocoon production in the range-finding test with TNB was significantly reduced at 10 mg kg⁻¹ ($p < 0.05$). There were no cocoons produced at 500 mg kg⁻¹ and no adults above 500 mg kg⁻¹. Results of these range-finding tests were used to determine treatment concentrations for the definitive tests.

3.3

Definitive Toxicity Tests.

Definitive studies using the Earthworm Reproduction Tests⁵ were conducted to assess the effects of RDX, HMX, 2,4-DNT, 2,6-DNT, or TNB on *E. fetida*. Adult *E. fetida* were exposed to a range of concentrations of each EM in SSL soil in independent investigations. Measurement endpoints were assessed using treatment concentrations determined using the results of the range-finding studies and included the number of surviving adults after 28 days, and the number of cocoons and juveniles after 56 days. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

Test results complied with the validity criteria defined in the ISO test guideline. Mean adult survival in negative controls was >90% in all tests. The coefficient of variation juvenile production in control treatments did not exceed 30%. Direct comparisons of the results of the positive control are not possible because no reference values for natural soils are available from the literature. Juvenile production in positive controls ranged from 54 to 98% reduction compared with negative controls and was within the baseline established for the laboratory culture of *E. fetida*. These results confirmed that the toxicological effects determined in the definitive tests were most likely due to test EM treatments. All reported ecotoxicological parameters have been calculated based on actual measured concentrations.

Results of toxicity testing in SSL soils with both freshly amended and weathered/aged RDX are shown in Tables 12 and 13, respectively. Adult *E. fetida* survival was not affected in all RDX concentrations producing unbounded NOEC values for RDX in freshly amended soils of 148.3 mg kg⁻¹ based on total concentrations and 93.5 mg kg⁻¹ based on ATCLP extractable concentrations. The unbounded NOEC value for weathered/aged RDX in amended soils based on total concentrations was 527.0 mg kg⁻¹. The unbounded NOEC value for weathered/aged RDX in amended soils based on ATCLP extractable concentrations was 100.1 mg kg⁻¹.

Cocoon production bounded NOEC and LOEC values based on total RDX concentrations were, 8.6 and 18.2 mg kg⁻¹ in freshly amended soil, and 56.6 and 61.5 mg kg⁻¹ weathered/aged RDX in soil, respectively (Table 14). Juvenile production bounded NOEC and LOEC values based on total concentrations were 7.5 and 8.6 mg kg⁻¹ in freshly amended soil, and 8.4 and 15.7 mg kg⁻¹ in weathered/aged RDX in soil, respectively (Table 14). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 2.1 and 5.2 mg kg⁻¹, and 13.6 and 30.0 mg kg⁻¹ in weathered/aged RDX in soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 2.1 and 5.2, and 5.8 and 13.6 mg kg⁻¹ in weathered/aged RDX in soils, respectively (Table 14).

Table 12. Mean (n = 4) adult survival, cocoon production, and juvenile production (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in SSL soils freshly amended with RDX; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Mean Hatched Cocoons (%)	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	9.3	1.5	71	23.6	12.8	5.1
Acetone control	5	0.0	13.3	0.3	83	3.7	18.3	1.1
Positive control	5	0.0	10.8	1.7	68	2.6	7.8	0.2
3	5	0.0	7.8	1.3	85	10.1	14.5	2.9
5	5	0.0	5.0	1.4	83	10.8	5.5	1.3
8	5	0.0	5.3	1.4	82	6.9	9.3	4.2
9	5	0.0	6.8	1.3	41	15.2	1.3	0.5
18	5	0.0	3.8	0.9	31	12.0	0.0	0.0
33	5	0.0	4.5	1.7	46	20.8	3.3	2.6
74	5	0.0	4.3	2.3	42	20.9	0.5	0.5
148	5	0.0	3.5	1.2	51	17.5	0.0	0.0

Table 13. Mean (n = 4) adult survival, cocoon production, and juvenile production (in weathered/aged RDX in amended SSL soils determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	4.8	0.3	17.5	1.6	58	23.6	17.3	2.7
Acetone control	4.8	0.3	18.3	1.3	54	3.7	24.3	7.5
Positive control	5.0	0.0	9.0	0.6	34	2.6	3.5	1.2
6	5.0	0.0	18.8	1.0	35	2.9	10.0	2.5
8	4.8	0.3	15.5	1.1	32	1.3	7.5	4.3
16	5.0	0.0	17.8	2.7	30	4.2	6.3	2.2
30	4.8	0.3	13.0	1.1	41	0.5	10.0	1.5
57	5.0	0.0	16.3	1.4	22	0.0	2.5	0.6
62	5.0	0.0	12.0	3.5	36	2.6	6.5	2.5
254	5.0	0.0	8.8	1.3	53	0.5	4.8	3.3
527	5.0	0.0	9.3	1.2	23	0.0	0.5	0.3

Concentration-response relationships for juvenile production in fresh and weathered/aged RDX in amended soils determined by nonlinear regressions are shown in Figure 1. Data fit the exponential model best in tests with both freshly amended (Figure 1A) and weathered/aged RDX in amended (Figure 1B) soils. Overall, reproduction was higher in weathered/aged RDX in amended soils (Table 13). Juvenile production EC₂₀ values based on total extraction were 1.6 and 4.8 mg kg⁻¹ in freshly amended and weathered/aged RDX in soils, respectively. Juvenile production EC₅₀ values were 5.0 and 14.9 mg kg⁻¹ for freshly amended and weathered/aged RDX in soils, respectively. Cocoon production EC₂₀ values were 1.2 and 19.2 mg kg⁻¹ for freshly amended and weathered/aged RDX in soil, respectively (Table 14). Cocoon production EC₅₀ values were 3.7 and 59.6 mg kg⁻¹ for freshly amended and weathered/aged RDX in soil, respectively (Table 14). Juvenile production EC₂₀ values based on ATCLP extractable concentrations were 0.84 and 1.4 mg kg⁻¹ for freshly amended and weathered/aged RDX in soils, respectively (Table 14). Juvenile production EC₅₀ values based on ATCLP extractable concentrations were 2.6 and 14.4 mg kg⁻¹ for freshly amended and weathered/aged RDX in soils, respectively (Table 16). The differences between freshly amended and weathered/aged RDX on cocoon and juvenile production were not statistically significant (95% CI; Table 14), indicating that the 3-month weathering/aging process did not affect the toxicity of RDX to *E. fetida*.

Coefficients of Determination (R²) for total and ATCLP based extractions of RDX were calculated in nonlinear regression analyses (EC₂₀ levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R² values for juveniles in freshly amended soil were 0.84 and 0.83 in total and ATCLP based

extractions, respectively (Table 14). The R^2 values for cocoons in freshly amended soil were 0.094 and 0.86 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.80 and 0.82 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in freshly amended soil were 0.95 and 0.95 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing RDX bioavailability to *E. fetida*.

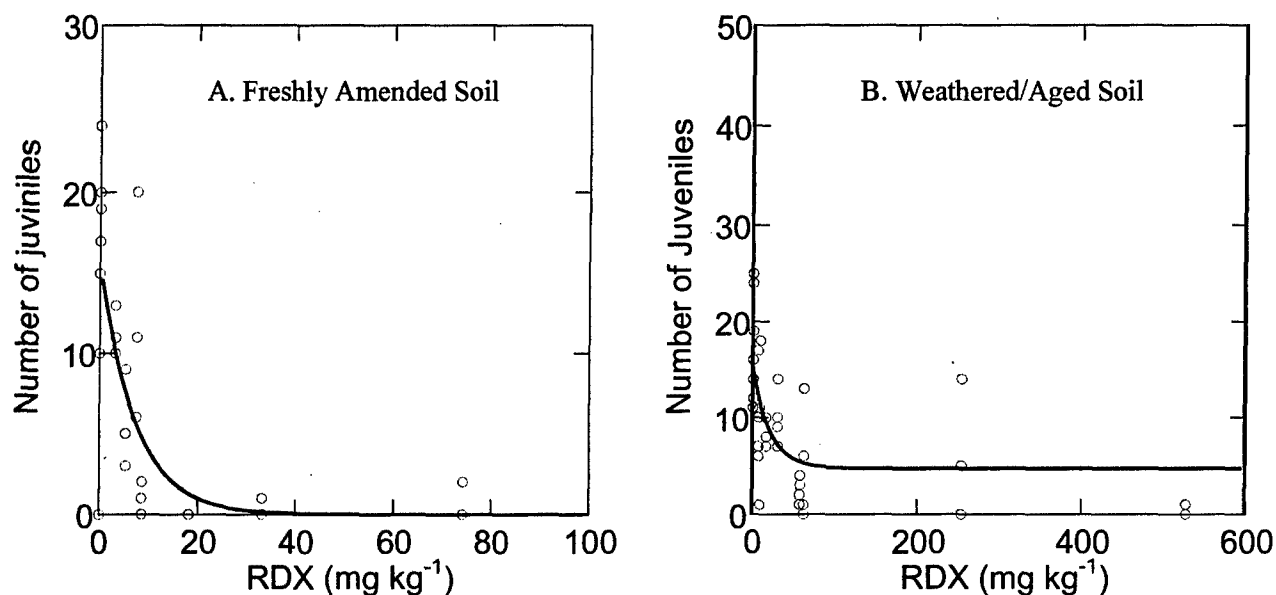


Figure 1. Non-linear regression [Exponential model: $Y = a \times e^{([log(1-p)] / ECp) \times C} + b$] of RDX and *Eisenia fetida* juvenile production (in freshly amended (A) and weathered/aged RDX amended (B) SSL soil; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Table 14. Ecotoxicological parameters (mg kg^{-1}) with p-value or confidence interval (C.I.) and coefficient of determination (R^2) for RDX (in freshly amended and weathered/aged RDX amended SSL soil using earthworm reproduction test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC ₂₀	EC ₅₀	NOEC	LOEC	EC ₂₀	EC ₅₀
Fresh								
Total	8.6	18.2	1.2	3.7	7.5	8.6	1.6	5.0
P or 95% C.I.	0.06	0.001	0.4-2.0	1.2-6.2	0.31	0.001	0.4-2.7	1.4-8.5
R ²			0.94	0.94			0.84	0.84
ATCLP	2.1	5.2	0.46	1.4	2.1	5.2	0.84	2.6
P or 95% C.I.	0.06	0.001	.031-.89	0.1-2.8	0.19	0.0001	0.35-1.3	1.1-4.1
R ²			0.86	0.86			0.83	0.83
Aged/weathered								
Total	56.6	61.5	19.2	59.6	8.4	15.7	4.8	14.9
P or 95% C.I.	0.45	0.01	0-39	0-120	0.06	0.02	0.2-9	0.66-29
R ²			0.95	0.95			0.80	0.80
ATCLP	13.6	30.0	42.0	102.7	5.8	13.6	1.4	14.4
P or 95% C.I.	0.95	0.03	11-73	67-139	0.61	0.01	0-5	0-31
R ²			0.95	0.95			0.82	0.82

p-value was generated by ANOVA.

C.I. and R^2 were generated by nonlinear regression analysis.

The results of HMX toxicity testing in freshly amended and weathered/aged HMX amended SSL soils are shown in Tables 15 and 16. Adult *E. fetida* survival was not affected in all HMX concentrations producing unbounded NOEC values for HMX in freshly amended soils of 141.3 mg kg^{-1} based on total concentrations and 15.2 mg kg^{-1} based on ATCLP extractable concentrations. The unbounded NOEC value for HMX in weathered/aged-amended soils based on total concentrations was 561.7 mg kg^{-1} . The unbounded NOEC value for HMX in weathered/aged-amended soils based on ATCLP extractable concentrations was 19.0 mg kg^{-1} .

Cocoon production bounded NOEC and LOEC values for HMX based on total concentrations were 15.6 and 36.0 mg kg^{-1} in freshly amended soil (Table 17). The cocoon production unbounded NOEC for weathered/aged HMX in soil was 561.7 mg kg^{-1} . Cocoon production was not significantly reduced ($p > 0.46$) in earthworm populations exposed to weathered/aged HMX in soil (Table 17). However, cocoon counts were reduced by 7 to 26% in treated soils compared with controls (Table 16). Juvenile production bounded NOEC and LOEC values based on total concentrations were 6.5 and 11.2 mg kg^{-1} in freshly amended soil (Table 17). The juvenile production unbounded NOEC for weathered/aged HMX in soil was 561.7 mg kg^{-1} . Juvenile production was not significantly reduced ($p > 0.59$) for earthworm populations exposed to total weathered/aged HMX in soil (Table 17). However, juvenile counts were reduced by 2 to 35% in treated soils compared with controls (Table 16). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 5.9 and

11.2 mg kg⁻¹, respectively. The ATCLP based cocoon production unbounded NOEC for weathered/aged HMX in soil was 19.0 mg kg⁻¹ ($p > 0.23$) (Table 17). The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 5.9 and 11.2 mg kg⁻¹ respectively. The ATCLP based juvenile production unbounded NOEC for weathered/aged HMX in soil was 19.0 mg kg⁻¹ ($p > 0.68$) (Table 17).

Table 15. Mean (n = 4) adult survival, cocoon production, and juvenile production (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in SSL soils freshly amended with HMX; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5	0.0	9.3	1.5	71	23.6	12.8	5.1
Acetone control	5	0.0	8.3	1.2	95	2.9	14.0	3.8
Positive control	5	0.0	3.0	1.2	65	23.6	0.3	0.3
1	5	0.0	7.8	1.7	75	9.0	8.3	1.3
3	5	0.0	4.8	2.4	89	7.9	6.3	3.2
7	5	0.0	7.0	0.7	81	8.9	7.3	4.1
11	5	0.0	5.3	1.1	50	20.4	4.0	2.3
16	5	0.0	5.5	1.0	70	14.9	5.0	2.2
36	5	0.0	2.8	0.9	82	10.7	2.8	1.8
74	5	0.0	3.5	1.0	79	15.8	4.8	2.3
141	5	0.0	4.5	1.3	92	4.9	4.3	2.6

Table 16. Mean (n = 4) adult survival and juvenile production and in weathered/aged HMX amended SSL soils (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	20.3	1.3	70	6.2	37.5	3.4
Acetone control	5.0	0.0	20.3	1.0	70	4.3	34.0	4.4
Positive control	5.0	0.0	9.0	0.8	34	7.7	3.5	1.2
2	5.0	0.0	15.0	1.7	63	13.6	21.8	7.9
3	5.0	0.0	15.3	1.7	57	7.6	22.0	6.0
11	5.0	0.0	18.5	3.3	62	11.9	33.5	12.5
29	5.0	0.0	16.8	0.6	53	7.8	24.5	6.6
54	5.0	0.0	18.8	2.8	60	5.1	31.3	5.7
129	5.0	0.0	15.8	2.3	64	8.4	29.0	6.7
280	5.0	0.0	15.0	2.8	61	18.0	27.3	8.4
562	5.0	0.0	18.3	2.8	54	7.5	26.0	2.1

Concentration-response relationships for juvenile production in fresh and weathered/aged HMX amended soils determined by nonlinear regressions are shown in Figure 2. Data fit the exponential model best in tests with both freshly amended (Figure 2A) and weathered/aged amended (Figure 2B) soils. Overall, reproduction was higher in weathered/aged HMX amended soils (Tables 15 and 16). Juvenile production EC₂₀ and EC₅₀ values based on total extraction were 0.4 and 1.2 mg kg⁻¹, respectively in freshly amended soil. Cocoon production EC₂₀ and EC₅₀ values based on total extraction were 2.7 and 8.5 mg kg⁻¹, respectively, in freshly amended soil (Table 17). Juvenile production EC₂₀ and EC₅₀ values based on ATCLP extraction were 0.08 and 0.25 mg kg⁻¹, respectively in freshly amended soil. Cocoon production EC₂₀ and EC₅₀ values based on total extraction were 1.4 mg kg⁻¹ and 4.3 mg kg⁻¹, respectively, in freshly amended soil (Table 17). Toxicity data in the HMX weathered/aged studies produced a nearly straight horizontal line (Figure 2B) indicating no effect. Therefore, EC₂₀ and EC₅₀ values could not be calculated for toxicological endpoints in *E. fetida* exposed to weathered/aged HMX in soil. The weathering/aging process virtually eliminated the toxicity of HMX to *E. fetida*. The extent of this effect could not be directly quantified since EC values could not be calculated. Reduced toxicity could not be explained by the degradation of HMX over time since an average of 75% of the HMX in the total extract was still present after weathering/aging (data not shown). ATCLP extractable HMX actually increased by an average of 124% after aging and weathering.

The R^2 values for juveniles in freshly amended soil were 0.0.74 and 0.73 in total and ATCLP based extractions, respectively (Table 17). The R^2 values for cocoons in freshly amended soil were 0.82 and 0.81 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing HMX bioavailability to *E. fetida*.

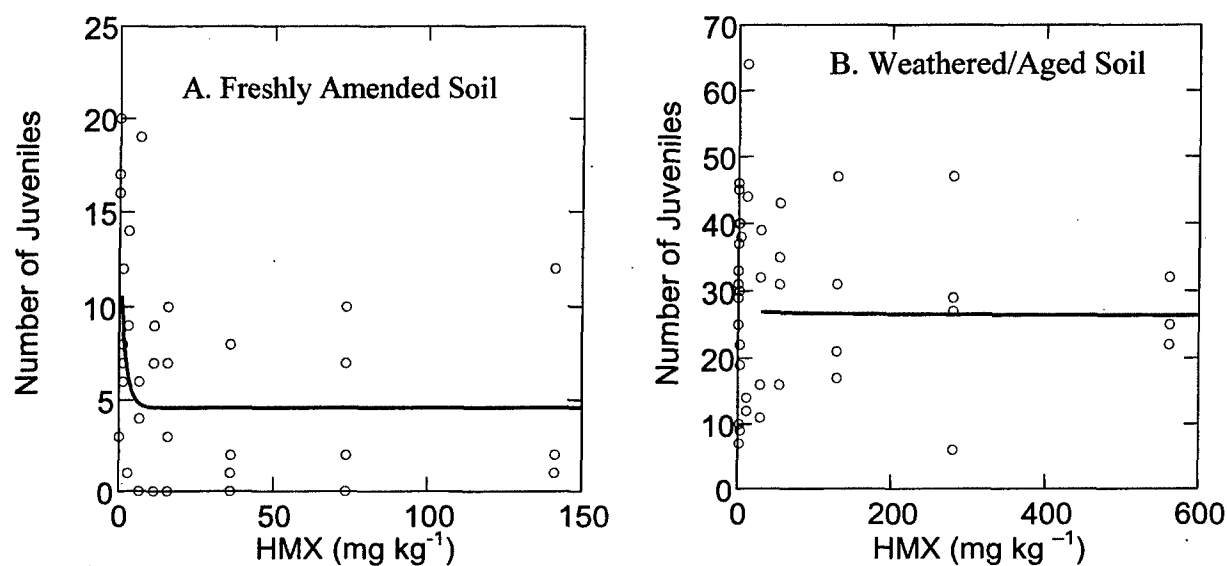


Figure 2. Non-linear regression [Exponential model: $Y = a \times e^{(([\log(1-p)] / EC_p) \times C)} + b$] of HMX and *Eisenia fetida* juvenile production (in freshly amended (A) and weathered/aged HMX in amended (B) SSL soil; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

The EM, 2,4-DNT affected adult survival, cocoon production, and juvenile production of *E. fetida* in amended SSL soil (Tables 18 and 19). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,4-DNT based on total concentrations were 55.0 and 64.7 mg kg⁻¹, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 33.4 and 43.4 mg kg⁻¹, respectively. For adult survival in weathered/aged 2,4-DNT in amended soil, the bounded NOEC and LOEC values based on total concentrations were 37.3 and 71.7 mg kg⁻¹, respectively. No adults survived in the 179 mg kg⁻¹ treatment. The bounded NOEC and LOEC values based on ATCLP concentrations were 20.5 and 46.1 mg kg⁻¹, respectively.

Cocoon production bounded NOEC and LOEC values based on total concentrations were 20.3 and 40.9 mg kg⁻¹ in freshly amended soil, and 21.5 and 31.0 mg kg⁻¹ in weathered/aged 2,4-DNT in soil, respectively (Table 20). Juvenile production bounded NOEC and LOEC values based on total concentrations were 55.0 and 64.7 mg kg⁻¹ in freshly amended soil, and 37.3 and 71.7 mg kg⁻¹ for weathered/aged 2,4-DNT in soil, respectively (Table 20). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 5.0 and 8.1 mg kg⁻¹, and 21.5 and 31.0 mg kg⁻¹ for weathered/aged 2,4-DNT in soil, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly

amended soils were 8.1 and 33.4, and 20.4 and 46.1 mg kg⁻¹ for weathered/aged 2,4-DNT in soil, respectively.

Table 17. Ecotoxicological parameters (mg kg⁻¹) with p-value or confidence interval (C.I.) and coefficient of determination (R²) for HMX (in freshly amended and weathered/aged HMX in amended SSL soil using earthworm reproduction test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC ₂₀	EC ₅₀	NOEC	LOEC	EC ₂₀	EC ₅₀
Fresh								
Total	15.6	36.0	2.7	8.5	6.5	11.2	0.4	1.2
P or 95% C.I.	.16	.007	0-7.0	0-22	0.1	0.02	0-0.9	0.5-2.8
R ²			0.82	0.82			0.74	0.74
ATCLP	5.9	11.2	1.4	4.3	5.9	11.2	.08	0.25
P or 95% C.I.	0.13	0.003	0-5.2	0-16	0.09	0.02	0-0.2	0-0.9
R ²			0.81	0.81			0.73	0.73
Aged/weathered								
Total	561.7	ND	ND	ND	561.7	ND	ND	ND
P or 95% C.I.	0.46				0.59			
R ²								
ATCLP	19.0	ND	ND	ND	19.0	ND	ND	ND
P or 95% C.I.	0.23				0.68			
R ²								

ND: Not Determined. ECx values could not be determined because cocoon and juvenile numbers were not significantly different in all treatment concentrations compared with carrier control. p-value was generated during ANOVA. C.I. and R² were generated during nonlinear regression analysis.

Table 18. Mean (n = 4) adult survival, cocoon production, and juvenile production (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in SSL soils freshly amended with 2,4-DNT; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5.0	0.0	15.0	2.3	44	11.0	5.5	2.5
Acetone control	4.8	0.3	12.5	0.5	52	16.0	7.0	3.7
Positive control	5.0	0.0	6.5	1.6	36	13.1	3.0	1.9
0.95	4.8	0.3	13.3	1.5	60	16.2	12.8	5.6
3.0	5.0	0.0	11.0	2.3	46	7.0	2.5	1.0
6.5	4.5	0.3	13.3	2.9	28	5.6	3.3	2.0
10.0	5.0	0.0	12.8	1.7	45	11.5	10.0	3.4
20.3	4.3	0.3	14.0	2.7	60	9.5	2.5	0.6
40.9	5.0	0.0	6.5	0.6	35	2.5	5.0	1.7
55.0	4.8	0.3	3.3	0.5	48	8.6	2.0	1.1
64.7	3.0	0.4	0.8	0.5	13	12.5	0.3	0.3

Table 19. Mean (n = 4) adult survival and juvenile production and in weathered/aged 2,4-DNT amended SSL soils (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	23.5	1.8	71.7	13.3	46.3	9.9
Acetone control	5.0	0.0	20.5	4.5	45.3	17.3	13.0	1.0
Positive control	3.5	0.0	2.8	1.0	43.3	20.8	0.8	0.5
3.0	5.0	0.0	18.8	2.3	79.7	6.3	45.3	12.9
5.2	5.0	0.0	17.3	3.8	34.7	13.1	9.8	4.8
11.5	4.8	0.0	22.0	3.2	61.3	9.6	43.5	7.6
22.0	5.0	0.0	17.5	1.0	69.9	10.6	45.8	5.4
31.0	4.8	0.0	10.3	3.5	48.6	16.7	20.0	7.6
37.0	4.8	0.0	12.8	4.0	69.0	7.7	19.0	5.1
72.0	4.0	0.0	1.3	0.5	27.5	12.5	0.3	0.3
179.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Concentration-response relationships for juvenile production in fresh and weathered/aged 2,4-DNT in amended soils determined by nonlinear regressions are shown in

Figure 3. The logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 3A) and weathered/aged 2,4-DNT in amended (Figure 3B) soils. Overall, reproduction was higher in weathered/aged 2,4-DNT in amended soils. Juvenile production EC_{20} values based on total extraction were 43.6 and 29.4 $mg\ kg^{-1}$ in freshly amended and weathered/aged 2,4-DNT in soil, respectively. Juvenile production EC_{50} values were 51.8 and 35.7 $mg\ kg^{-1}$ in freshly amended and weathered/aged 2,4-DNT in soil, respectively. Cocoon production EC_{20} values were 30.7 and 25.2 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged 2,4-DNT in soil, respectively (Table 20). Cocoon production EC_{50} values were 42.9 and 40.5 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged 2,4-DNT in soil, respectively (Table 20). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 22.2, and 29.4 $mg\ kg^{-1}$ in freshly amended and weathered/aged 2,4-DNT in soil, respectively (Table 20). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 29.6 and 19.2 $mg\ kg^{-1}$ in freshly amended and weathered/aged 2,4-DNT in soil, respectively (Table 20). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 5.3 and 12.3 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged soil, respectively (Table 20). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 14.3 and 22.3 $mg\ kg^{-1}$ in freshly amended soil and for weathered/aged 2,4-DNT in soil, respectively (Table 20). The differences between freshly amended and weathered/aged 2,4-DNT for cocoon and juvenile production were not statistically significant (95% CI; Table 20), indicating that the 3-month weathering/aging process did not affect the toxicity of 2,4-DNT to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of 2,4-DNT were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2 values for juveniles in freshly amended soil were 0.63 and 0.57 in total and ATCLP based extractions, respectively (Table 20). The R^2 values for cocoons in freshly amended soil were 0.094 and 0.93 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.81 and 0.84 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged soil were 0.93 and 0.94 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing 2,4-DNT bioavailability to *E. fetida*.

The EM, 2,6-DNT affected adult survival, cocoon production, and juvenile production of *E. fetida* in amended SSL (Tables 21 and 22). For adult survival in freshly amended soil, the bounded NOEC and LOEC values for 2,6-DNT based on total concentrations were 20.0 and 40.2 $mg\ kg^{-1}$, respectively. The bounded NOEC and LOEC values based on ATCLP concentrations were 11 and 25 $mg\ kg^{-1}$, respectively. For adult survival in weathered/aged 2,6-DNT in amended soil, the bounded NOEC and LOEC values based on total concentrations were 13.9 and 18.0 $mg\ kg^{-1}$, respectively. No adults survived in the 179 $mg\ kg^{-1}$ treatment. The bounded NOEC and LOEC values for adult survival in weathered/aged 2,6-DNT in soil based on ATCLP concentrations were 6.6 and 9.6 $mg\ kg^{-1}$, respectively.

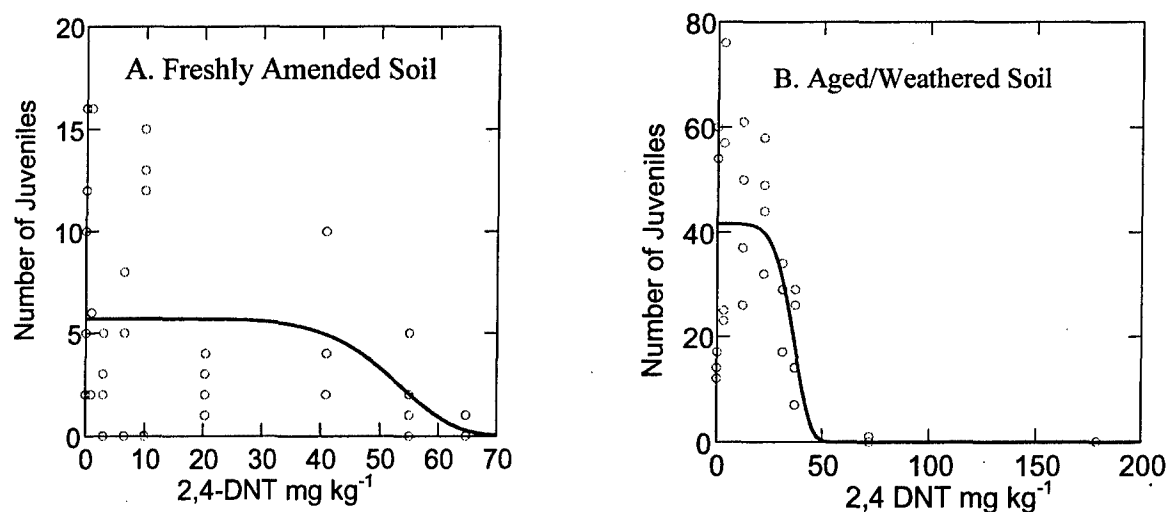


Figure 3. Non-linear regression [Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)] \times [C/EC_p]b)}$ of 2,4-DNT and *Eisenia fetida* juvenile production (in freshly amended (A) and weathered/aged (B) 2,4-DNT in amended SSL soil; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Table 20. Ecotoxicological parameters (mg kg⁻¹) with p-value or confidence interval (C.I.) and coefficient of determination (R^2) for 2,4-DNT (in freshly amended and weathered/aged amended SSL soil using earthworm reproduction test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC ₂₀	EC ₅₀	NOEC	LOEC	EC ₂₀	EC ₅₀
Fresh								
Total	20.3	40.9	30.7	42.9	55.0	64.7	43.6	51.8
<i>P</i> or 95% C.I.	0.91	0.003	17.4-44.1	34.3-51.6	.066	.021	10.7-76.5	33.2-70.4
R^2			0.94	0.94			0.63	0.63
ATCLP	5.0	8.1	5.3	14.3	8.1	33.4	22.2	29.6
<i>P</i> or 95% C.I.	0.65	0.003	0-10.7	5.8-22.8	0.23	.006	-34.2-78.6	0.1-59.0
R^2			0.91	0.91			0.57	0.57
Aged/weathered								
Total	21.5	31.0	25.2	40.5	37.3	71.7	29.4	35.7
<i>P</i> or 95% C.I.	0.1	0.01	15.9-34.5	31.6-49.4	0.12	0.002	17.4-41.5	30.0-41.5
R^2			0.93	0.93			0.81	0.81
ATCLP	11.8	15.4	12.3	22.3	20.4	46.1	15.2	19.2
<i>P</i> or 95% C.I.	0.10	0.001	6.7-17.9	16.1-28.4	0.5	0.001	8.1-22.3	15.2-23.3
R^2			0.94	0.94			0.84	0.84

p-value was generated during ANOVA.

C.I. and R^2 were generated during nonlinear regression analysis.

Cocoon production bounded NOEC and LOEC values based on total concentrations were 9.4 and 12.9 mg kg⁻¹ in freshly amended soil, and 18.1 and 37.4 mg kg⁻¹ in weathered/aged soil, respectively (Table 23). Juvenile production bounded NOEC and LOEC values based on total concentrations were 20.0 and 40.2 mg kg⁻¹ in freshly amended soil, and 13.9 and 18.1 mg kg⁻¹ in weathered/aged soil, respectively (Table 23). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 3.8 and 5.8 mg kg⁻¹, and 9.6 and 17.4 mg kg⁻¹ in weathered/aged soils, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 10.6 and 24.8, and 6.6 and 9.6 mg kg⁻¹ in weathered/aged soils, respectively (Table 23).

Table 21. Mean (n = 4) adult survival, cocoon production, and juvenile production (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in SSL soils freshly amended with 2,6-DNT; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5.0	0.0	15.0	2.3	44	11.0	5.5	2.5
Acetone control	4.8	0.3	12.5	0.5	52	16.0	7.0	3.7
Positive control	5.0	0.0	6.5	1.6	36	13.1	3.0	1.9
5.0	4.8	0.3	13.3	1.5	60	16.2	12.8	5.6
7.7	5.0	0.0	11.0	2.3	46	7.0	2.5	1.0
9.4	4.5	0.3	13.3	2.9	28	5.6	3.3	2.0
13.0	5.0	0.0	12.8	1.7	45	11.5	10.0	3.4
20.0	4.3	0.3	14.0	2.7	60	9.5	2.5	0.6
40.0	5.0	0.0	6.5	0.6	35	2.5	5.0	1.7
51.0	4.8	0.3	3.3	0.5	48	8.6	2.0	1.1
64.0	3.0	0.4	0.8	0.5	13	12.5	0.3	0.3

Table 22. Mean (n = 4) adult survival and juvenile production and in weathered/aged 2,6-DNT amended SSL soils (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	11.8	1.4	62	8.1	14.3	2.7
Acetone control	5.0	0.0	13.0	4.4	75	12.5	25.3	11.5
Positive control	5.0	0.0	9.0	1.4	43	18.8	5.8	3.2
1.2	5.0	0.0	18.8	5.2	65	17.0	29.0	11.2
1.6	4.3	1.1	12.3	4.5	65	16.0	23.8	13.8
3.7	5.0	0.0	16.3	2.1	52	15.4	17.8	6.7
9.5	5.0	0.0	20.0	3.8	45	11.1	18.0	8.1
13.9	5.0	0.0	13.5	2.7	40	15.5	5.0	2.8
18.0	3.3	1.7	10.3	3.5	15	9.8	0.8	0.8
37.0	1.5	1.4	0.0	0.0	0	0.0	0.0	0.0
108.0	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0

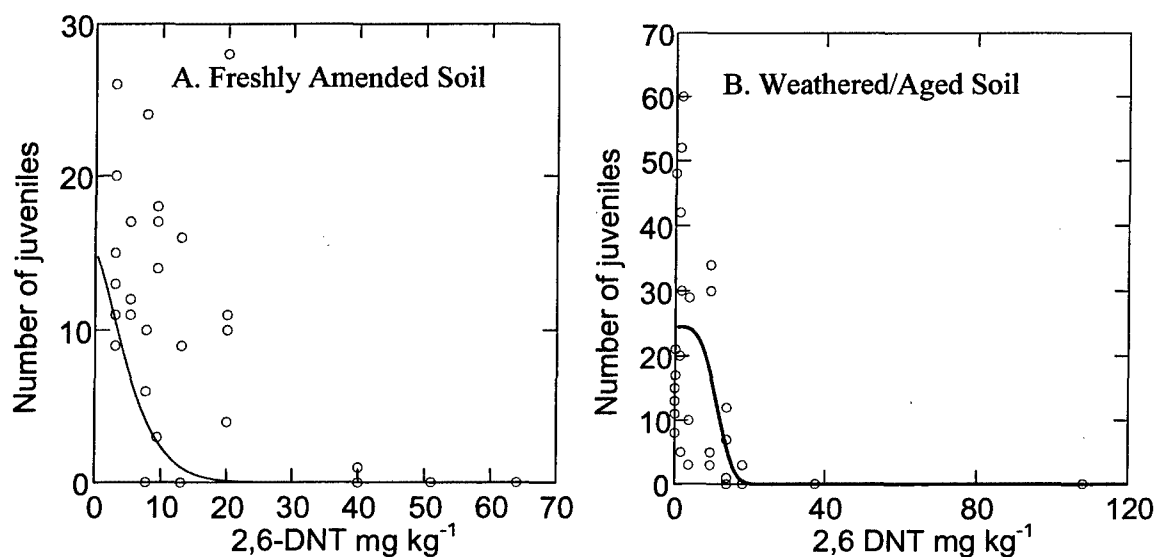


Figure 4. Non-linear regression [Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)] \times [C/EC_p]b)}$] of 2,6-DNT and *Eisenia fetida* juvenile production (in freshly amended (A) and weathered/aged 2,6-DNT in amended (B) SSL soil; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Concentration-response relationships for juvenile production in fresh and weathered/aged 2,6-DNT in amended soils determined by nonlinear regressions are shown in Figure 4. The logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 4A) and weathered/aged 2,6-DNT in amended (Figure 4B) soils. Overall, reproduction was higher in weathered/aged 2,6-DNT in amended soils. Juvenile production EC_{20} values based on total extraction were 9.0 and 8.3 mg kg⁻¹ in freshly amended and weathered/aged 2,6-DNT in soil, respectively (Table 23). Juvenile production EC_{50} values were 27.4 and 11.3 mg kg⁻¹ in freshly amended and weathered/aged 2,6-DNT in soil, respectively. Cocoon production EC_{20} values were 14.3 and 16.1 mg kg⁻¹ in freshly amended soil and in weathered/aged 2,6-DNT in soil, respectively (Table 23). Cocoon production EC_{50} values were 24.8 and 19.3 mg kg⁻¹ in freshly amended soil and in weathered/aged 2,6-DNT in soil, respectively (Table 23). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 6.5, and 3.6 mg kg⁻¹ in freshly amended and weathered/aged 2,6-DNT in soils, respectively (Table 23). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 20.2 and 5.2 mg kg⁻¹ in freshly amended and weathered/aged 2,6-DNT in soil, respectively (Table 23). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 7.4 and 16.1 mg kg⁻¹ in freshly amended soil and in weathered/aged 2,6-DNT in soil, respectively (Table 23). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 14.2 and 10.5 mg kg⁻¹ in freshly amended 2,6-DNT in soil and in weathered/aged soil, respectively (Table 23). The differences between freshly amended and weathered/aged 2,6-DNT on cocoon and juvenile production were not statistically significant (95% CI; Table 22), indicating that the 3-month weathering/aging process did not affect the toxicity of 2,6-DNT to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of 2,6-DNT were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged 2,6-DNT in soil. The R^2 values for juveniles in freshly amended soil were 0.71 and 0.73 in total and ATCLP based extractions, respectively (Table 23). The R^2 values for cocoons in freshly amended soil were 0.92 and 0.91 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged 2,6-DNT in soil were 0.72 and 0.67 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged 2,6-DNT in soil were 0.91 and 0.85 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing 2,6-DNT bioavailability to *E. fetida*.

Table 23. Ecotoxicological parameters (mg kg^{-1}) with p-value or confidence interval (C.I.) and coefficient of determination (R^2) for 2,6-DNT (in freshly amended and weathered/aged 2,6-DNT in amended SSL soil using earthworm reproduction test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC ₂₀	EC ₅₀	NOEC	LOEC	EC ₂₀	EC ₅₀
Fresh								
Total	9.4	12.9	14.3	24.8	20.0	40.2	9.0	27.4
P or 95% C.I.	.545	.035	6.6-22.1	17.4-32.1	.56	.001	0-30.5	0-91.8
R ²			0.92	0.92			0.71	0.71
ATCLP	3.8	5.8	7.4	14.2	10.6	24.8	6.5	20.2
P or 95% C.I.	.55	.035	2.9-11.9	9.3-19.1	.56	.001	0-23.8	0-74.0
R ²			0.93	0.93			0.73	0.73
Aged/weathered								
Total	18.1	37.4	16.1	19.3	13.9	18.1	8.3	11.3
P or 95% C.I.	0.58	0.002	9.9-22.2	13.4-25.3	0.09	0.03	1.6-15.1	6.9-15.8
R ²			0.91	0.91			0.71	0.71
ATCLP	9.6	17.4	8.2	10.5	6.6	9.6	3.6	5.2
P or 95% C.I.	0.58	0.002	3.9-12.6	6.2-14.7	0.09	0.03	0.04-7.2	2.8-7.6
R ²			0.85	0.85			0.67	0.67

p-value was generated during ANOVA. C.I. and R^2 were generated during nonlinear regression analysis.

Results of TNB toxicity testing in freshly amended and weathered/aged amended SSL soils are shown in Tables 24 and 25, respectively. For adult survival in freshly amended soil, the bounded NOEC and LOEC values for TNB based on total concentrations were 45 and 107 mg kg^{-1} , respectively (Table 26). The bounded NOEC and LOEC values based on ATCLP concentrations were 30 and 84 mg kg^{-1} , respectively (Table 26). For adult survival in weathered/aged 2,6-DNT in amended soil, the bounded NOEC and LOEC values based on total concentrations were 79 and 191 mg kg^{-1} , respectively. No adults survived in the 302 mg kg^{-1} treatment. The bounded NOEC and LOEC values for adults based on ATCLP TNB concentrations were 42 and 112 mg kg^{-1} , respectively.

Bounded NOEC and LOEC values for cocoon production based on total TNB concentrations were, 13.6 and 45.0 mg kg^{-1} in freshly amended soil, and 19.9 and 78.7 mg kg^{-1} in weathered/aged TNB in soil, respectively (Table 26). Bounded NOEC and LOEC values for juvenile production based on total concentrations were 13.6 and 45.0 mg kg^{-1} in freshly amended soil, and 19.9 and 78.7 mg kg^{-1} in weathered/aged TNB in soil, respectively (Table 26). The ATCLP based NOEC and LOEC values for cocoon production in freshly amended soils were 7.7 and 30.2 mg kg^{-1} , and 6.2 and 41.8 mg kg^{-1} for weathered/aged TNB in soil, respectively. The ATCLP based NOEC and LOEC values for juvenile production in freshly amended soils were 7.7 and 30.2, and 6.2 and 41.8 mg kg^{-1} for weathered/aged TNB in soils, respectively (Table 26).

Table 24. Mean (n = 4) adult survival, cocoon production, and juvenile production (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida* in SSL freshly amended with TNB; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean Cocoons	Standard Error	Percent Hatched Cocoons	Standard Error	Mean Juveniles	Standard Error
Negative control	5.0	0.0	14.0	1.0	63	14.8	11.3	4.1
Acetone control	4.8	0.3	13.5	1.7	50	15.4	11.0	4.2
Positive control	5.0	0.0	3.0	0.6	31	23.7	0.3	0.3
2.3	5.0	0.0	14.3	1.3	63	8.6	18.0	1.7
2.6	4.7	0.3	17.0	2.5	85	1.5	22.3	3.8
3.9	5.0	0.0	10.5	2.3	76	9.1	17.0	3.2
13.6	4.8	0.3	16.5	1.6	72	6.2	15.3	4.2
45.0	4.8	0.3	7.5	1.8	37	12.8	3.8	1.7
107.0	2.3	0.3	3.8	0.9	5	5.0	0.0	0.0
221.0	0.5	0.5	0.0	0.0	0	0.0	0.0	0.0
385.0	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0

Table 25. Mean (n = 4) adult survival and juvenile production and in weathered/aged TNB in amended SSL soils (determined in toxicity testing using Earthworm Reproduction Test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330. BDL = below method detection limit of 0.05 mg kg⁻¹).

Soil concentration (mg kg ⁻¹)	Mean Adults	Standard Error	Mean cocoons	Standard Error	Mean hatched cocoons (%)	Standard Error	Mean juveniles	Standard Error
Negative control	5.0	0.0	20.5	0.9	81	3.5	54.8	1.9
Acetone control	5.0	0.0	23.3	1.6	77	4.2	51.8	5.1
Positive control	5.0	0.0	3.0	0.6	31	23.7	0.3	0.3
BDL (nominal 16)	4.8	0.3	20.8	0.6	79	5.5	52.3	3.1
1	5.0	0.0	22.8	1.5	65	10.8	47.3	9.4
20	5.0	0.0	24.0	3.0	75	6.3	50.0	4.8
79	3.8	1.3	5.8	2.0	56	19.1	7.8	4.4
191	4.0	0.4	0.0	0.0	0	0.0	0.0	0.0
302	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0
411	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0
674	0.0	0.0	0.0	0.0	0	0.0	0.0	0.0

Concentration-response relationships for juvenile production in fresh and weathered/aged TNB in amended soils determined by nonlinear regressions are shown in Figure 5. The logistic (Gompertz) model had the best fit for data in tests with both freshly amended (Figure 5A) and weathered/aged TNB in amended (Figure 5B) soils. Overall, reproduction was higher in weathered/aged TNB amended soils. Juvenile production EC_{20} values based on total extraction were 21.4 and 13.2 $mg\ kg^{-1}$ in freshly amended and weathered/aged TNB in soil, respectively. Juvenile production EC_{50} values were 33.3 and 41.1 $mg\ kg^{-1}$ in freshly amended and weathered/aged soils, respectively. Cocoon production EC_{20} values were 27.2 and 18.2 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged soil, respectively (Table 26). Cocoon production EC_{50} values were 59.1 and 56.6 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged soil, respectively (Table 26). Juvenile production EC_{20} values based on ATCLP extractable concentrations were 6.6 and 5.8 $mg\ kg^{-1}$ in freshly amended and weathered/aged soils, respectively (Table 26). Juvenile production EC_{50} values based on ATCLP extractable concentrations were 20.6 and 18.0 $mg\ kg^{-1}$ in freshly amended and weathered/aged soils, respectively (Table 26). Cocoon production EC_{20} values based on ATCLP extractable concentrations were 13.4 and 8.4 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged soil, respectively (Table 26). Cocoon production EC_{50} values based on ATCLP extractable concentrations were 41.6 and 26.2 $mg\ kg^{-1}$ in freshly amended soil and in weathered/aged soil, respectively (Table 26). The differences between freshly amended and weathered/aged cocoon and juvenile production were not statistically significant (95% CI; Table 26), indicating that the 3-month weathering/aging process did not affect the toxicity of TNB to *E. fetida*.

Coefficients of Determination (R^2) for total and ATCLP based extractions of TNB were calculated in nonlinear regression analyses (EC_{20} levels) to determine which chemical measure better correlates with toxicity endpoints in both fresh and weathered/aged soils. The R^2 values for juveniles in freshly amended soil were 0.92 and 0.95 in total and ATCLP based extractions, respectively (Table 26). The R^2 values for cocoons in freshly amended soil were 0.94 and 0.96 in total and ATCLP based extractions, respectively. The R^2 values for juveniles in weathered/aged soil were 0.95 and 0.96 in total and ATCLP based extractions, respectively. The R^2 values for cocoons in weathered/aged soil were 0.96 and 0.97 in total and ATCLP based extractions, respectively. These comparisons show that regression coefficients were very similar for both extraction methods indicating that neither extraction method had an advantage in characterizing TNB bioavailability to *E. fetida*.

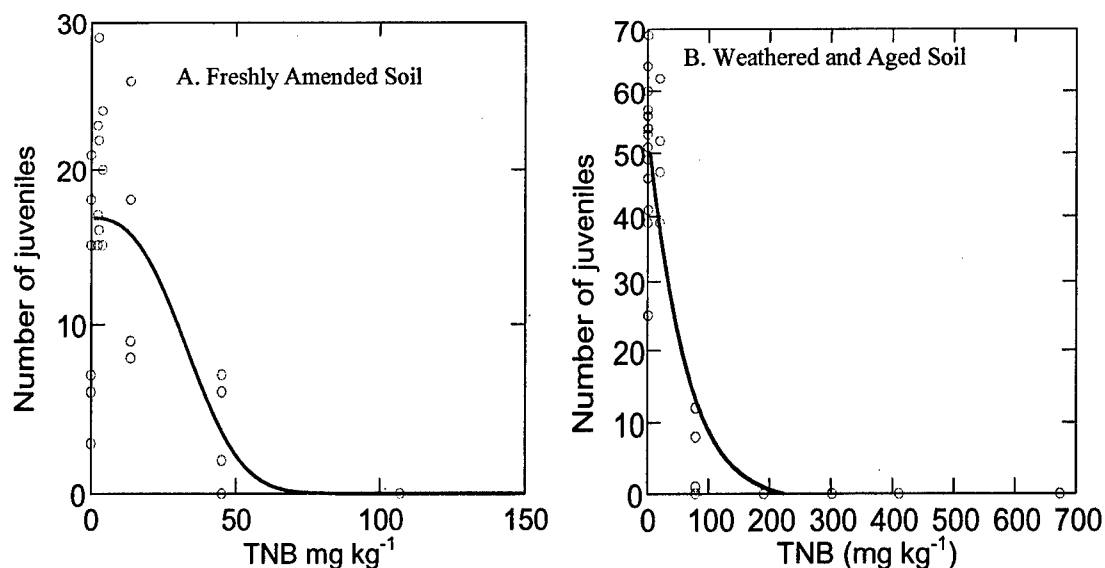


Figure 5. Non-linear regression [Logistic (Gompertz) model: $Y = a \times e^{([\log(1-p)] \times [C/EC_p]b)}$] of TNB and *Eisenia fetida* juvenile production (in freshly amended (A) and weathered/aged amended (B) SSL soil; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Table 26. Ecotoxicological parameters (mg kg⁻¹) with p-value or confidence interval (C.I.) and coefficient of determination (R²) for TNB (in freshly amended and weathered/aged amended SSL soil using earthworm reproduction test with *Eisenia fetida*; concentrations are based on acetonitrile extraction using U.S. EPA Method 8330).

Exposure	Cocoon Production				Juvenile production			
	NOEC	LOEC	EC ₂₀	EC ₅₀	NOEC	LOEC	EC ₂₀	EC ₅₀
Fresh								
Total	13.6	45.0	27.2	59.1	13.6	45.0	21.4	33.3
P or 95% C.I.	0.09	0.0001	6.5-48.0	37.0-81.2	0.23	0.04	0-55.2	9.0-57.5
R ²			0.94	0.94			0.92	0.92
ATCLP	7.7	30.2	13.4	41.6	7.7	30.2	6.6	20.6
P or 95% C.I.	.194	.0001	6.0-20.8	18.5-64.6	.99	.002	0-13.3	0-41.3
R ²			0.95	0.95			0.81	0.81
Aged/weathered								
Total	19.9	78.7	18.2	56.6	19.9	78.7	13.2	41.1
P or 95% C.I.	0.13	0.0001	10.7-25.8	33.1-80.1	0.52	0.0001	7.3-19.1	22.6-59.5
R ²			0.96	0.96			0.95	0.95
ATCLP	6.2	41.8	8.4	26.2	6.2	41.8	5.8	18.0
P or 95% C.I.	0.17	0.0001	5.1-11.8	15.8-36.6	0.7	0.0001	3.1-8.5	9.5-26.5
R ²			0.97	0.97			0.96	0.96

ND: Not Determined. EC_x values could not be determined because cocoon and juvenile numbers were not significantly different in all treatment concentrations compared with carrier control.

p-value was generated during ANOVA. C.I. and R² were generated during nonlinear regression analysis.

The present study supported the scientifically based ecological soil screening level (Eco-SSL) requirements for establishing benchmark screening concentration levels for soil contaminants. The majority of previously reported soil toxicity test results utilized standard artificial soil with high organic matter content (10%). In contrast, these toxicity studies used a natural soil that met the criteria for Eco-SSL development because its characteristics support relatively high bioavailability of energetic materials (EMs). Most of the previous studies measured only lethal endpoints. This study used reproductive as well as lethal endpoints. The results show that reproductive endpoints are much more sensitive indicators of toxicity. In addition, the soil weathering/aging procedure enabled a more realistic toxicity assessment of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), and 1,3,5-trinitrobenzene (TNB) under conditions more closely resembling those encountered in the field.

Definitive toxicity tests conducted with freshly amended soils showed that the order of EM toxicity, based on EC_{20} values for juvenile production with *Eisenia fetida*, was $HMX > RDX > 2,6-DNT > TNB > 2,4-DNT$. Definitive toxicity tests conducted with weathered/aged amended soils showed that the EM toxicity order based on EC_{20} values for juvenile production in *E. fetida* tests was $RDX > 2,6-DNT > TNB > 2,4-DNT > HMX$. The reproduction measurement endpoints in all the tests were more sensitive compared with adult survival.

In this study, both cocoon and juvenile production were reduced at relatively low levels of RDX and HMX in freshly amended soils. Juvenile production was affected by RDX with EC_{20} estimates of 1.6 and 5 $mg\ kg^{-1}$ in freshly amended and weathered/aged amended soils, respectively. However, some cocoons were still found at 148 $mg\ kg^{-1}$ in the definitive tests and 5000 $mg\ kg^{-1}$ in the range-finding tests. This may have been due to the low bioavailability of EMs in the soil. The RDX and HMX solubility in 20 °C water is 42.3 and 6.63 $mg\ L^{-1}$, respectively.¹⁷ The Adapted Toxicity Characteristic Leaching Procedure (ATCLP) extractable (and presumably bioavailable) fractions ranged from 100 to 18% of acetonitrile extractable concentration for RDX, and from >100 to 3% of acetonitrile extractable concentration for HMX. Adult *E. fetida* survival was not affected by RDX and HMX even at concentrations as high as 5,000 $mg\ kg^{-1}$ in range-finding tests. Weathering and aging of RDX amended soil did not significantly (95% Confidence Intervals (CI)) affect its toxicity to *E. fetida*.

The results of RDX and HMX toxicity tests found in this study may not directly compare to those of other studies since none of the latter were designed to meet the Eco-SSL criteria of testing for soil invertebrates. Literature on the toxicity of RDX to terrestrial organisms is scant, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Significant sub lethal effects of RDX were observed on the reproduction of the earthworm *E. andrei* at concentrations as low as 95 $mg\ kg^{-1}$ soil.¹⁸ However, mortality and reproduction of the enchytraeid worm *E. crypticus* and collembolan *Folsomia candida* in soils spiked with up to 1000 $mg\ kg^{-1}$ RDX in soil were not affected.¹⁹ Furthermore, these studies were conducted in either standard artificial soil,¹⁸ or in soil with relatively high (2.5 to 3.0%) organic carbon,¹⁹ which limits their usefulness for describing natural systems or the development of Eco-

SSLs. The bioavailability of nonpolar organic chemicals in soil is hypothesized to be determined primarily by soil organic matter (OM) content.²⁰ Sassafras sandy loam has 1.2% OM compared to the 10% in artificial soil. These authors also suggest that bioaccumulation and toxicity are well correlated with the concentration of chemical in the soil solution or pore water, rather than total chemical levels. In the present study, total extractable and water extractable RDX and HMX showed no difference in correlation to toxicity.

Of the 5 EM compounds tested in this study in freshly amended SSL soil (cocoon $EC_{20} = 2.7 \text{ mg kg}^{-1}$, juvenile $EC_{20} = 0.4 \text{ mg kg}^{-1}$), HMX was the most toxic to the *E. fetida* reproductive endpoints. The HMX toxicity was greatly reduced after the weathering and aging of the soil. Significant differences among means in Analysis of Variance (ANOVA) and the lack of fit regression models due to the lack of Lowest Observed Effect Concentration (LOEC), EC_{20} , and EC_{50} could not be calculated for HMX in weathered/aged soil. However, most of the HMX was still present in the acetonitrile fraction after weathering/aging (mean = 75%) and the mean HMX concentration in the ATCLP fraction actually increased slightly over time (128%). The cause of the decreased toxicity was beyond the scope of this study. The HMX could either have been in a different, less toxic form or it could have been too tightly bound to soil or organic matter in the aged soil. Further testing is required to elucidate the cause.

Among the nitroaromatic compounds evaluated in this study, 2,6-DNT was most toxic. Comparison of the results to other studies that evaluated toxicity of nitroaromatic compounds is difficult because the toxicity of nitroaromatic energetics, including 2,4-DNT, 2,6-DNT, and TNB, to soil invertebrates has not been sufficiently investigated. The majority of studies reported in the available literature focused primarily on the effects of 2,4,6-trinitrotoluene (TNT) and/or its degradation products.^{1,19,21-25} Phillips *et al.* reported 100% mortality in the earthworm *E. fetida* growth and survival test in standard artificial soil amended with a mixture of EMs that included 30, 50, 62.5, and 20 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT.²⁵ Statistically significant ($p < 0.01$) sub lethal effects (mass loss) were reported at 6, 10, 12.5, and 4 mg kg^{-1} of TNT, TNB, 2,4-DNT and 2,6-DNT. These results are similar to the findings in this study although direct comparisons of both studies are limited due to differences in the experimental designs.

Simini *et al.* assessed the toxicity of the soil from the Joliet Army Ammunition Plant contaminated with a mixture of EMs (which limits the direct comparisons with our study), including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays.¹ The highest soil concentrations measured at this site were 200, 117, and 8 mg kg^{-1} for TNB, 2,4-DNT and 2,6-DNT. The authors reported that linear regression of TNT and TNB data yielded the greatest coefficients of determination (R^2) in all bioassays, including the earthworm test. The R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for 2 locations investigated at the study site. These values were 0.613 and 0.358 for 2,4-DNT, whereas 2,6-DNT had the weakest relationship with measurement points with R^2 values of 0.082 and 0.293 for both locations. Soil TNB and 2,4-DNT concentrations found at this site were within the range of concentrations tested in this study and the results of both were consistent. The weak relationship between toxicity and 2,6-DNT was most likely due to very low concentrations of the EM measured at the site.

The weathering and aging procedure was incorporated more closely to stimulate the exposure effects on soil invertebrates in the field. The weathering and aging of RDX amended soil for 90 days did not reduce RDX concentrations or significantly affect its toxicity to *E. fetida*. However, the process rendered HMX non-toxic to earthworm reproduction even though soil concentration was not reduced. Further study is needed to elucidate the mechanisms responsible for reduced HMX toxicity in weathered/aged soils. Toxicity of 2,4-DNT, 2,6-DNT, and TNB was not altered by the weathering/aging process.

Specific mechanisms of changes in the toxicity of EMs in weathered/aged amended soil are unknown. In some cases, degradation products yielded during the weathering and aging process may be more toxic to soil organisms than the parent material. Dodard *et al.*²⁴ investigated the toxic effects of 2,4-DNT and 2,6-DNT, and their respective metabolites using the 15-min Microtox (*Vibrio fischeri*) and 96-hr freshwater green alga (*Selenastrum capricornutum*) growth inhibition tests. The toxicities of DNTs were species-dependent: 2,4-DNT was more toxic than 2,6-DNT to *S. capricornutum* (comports with the results for *E. crypticus*), while the reverse was true in the test with *Vibrio fischeri*. The authors reported that the reduced metabolites of 2,6-DNT tested were less toxic compared to the toxicity of the parent compound although certain partially reduced metabolites of 2,4-DNT (4-amino-2-nitrotoluene and 2-amino-4-nitrotoluene) were more toxic. Even though those results cannot be directly compared to the results reached in this study because the biotic reductive degradation pathway for 2,4-DNT and 2,6-DNT in aquatic environment would contrast with the metabolic processes in the aerobic conditions of vadose zone simulated in this investigation, the reducing environment can exist in water-logged soil microsites, where more toxic metabolites of dinitrotoluene degradation may be present.

The exposure concentrations of RDX, HMX, 2,4-DNT, 2,6-DNT, and TNB in soil were determined using both acetonitrile extraction (total chemical measure) and water extraction methods. The water extractable portion of each EM was determined using ATCLP to establish whether this technique, which is designed to measure the leachable, and presumably bioavailable fraction of chemicals in soil, could generate data that is better correlated with toxicity than total chemical measurement. Coefficients of determination (R^2), calculated by non linear regression analysis of acetonitrile extraction data, were compared to the R^2 values from ATCLP extraction data to determine which chemical measure of exposure better correlated with toxicity. These comparisons showed that R^2 values were similar in both exposure types. Therefore, neither extraction method had an advantage in characterizing toxicity of EMs tested to *E. fetida* in this study. This result supports the decision to develop Eco-SSLs for explosive contaminants in soil on the basis of the acetonitrile extraction of test compounds. Acetonitrile extraction-based Eco-SSLs will be especially useful for Ecological Risk Assessment (ERA) at contaminated sites because EM concentrations, determined during site characterization, are usually based on total extraction according to US EPA method 8330.

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